Accelerated homologous recombination and subsequent genome modification in Drosophila

Luis Alberto Baena-Lopez*, Cyrille Alexandre*, Alice Mitchell, Laurynas Pasakarnis and Jean-Paul Vincent‡

ABSTRACT
Gene targeting by ‘ends-out’ homologous recombination enables the deletion of genomic sequences and concurrent introduction of exogenous DNA with base-pair precision without sequence constraint. In Drosophila, this powerful technique has remained laborious and hence seldom implemented. We describe a targeting vector and protocols that achieve this at high frequency and with very few false positives in Drosophila, either with a two-generation crossing scheme or by direct injection in embryos. The frequency of injection-mediated gene targeting can be further increased with CRISPR-induced double-strand breaks within the region to be deleted, thus making homologous recombination almost as easy as conventional transgenesis. Our targeting vector replaces genomic sequences with a multifunctional fragment comprising an easy-to-select marker, a fluorescent reporter, as well as an attP site, which acts as a landing platform for reintegration vectors. These vectors allow the insertion of a variety of transcription reporters or cDNAs to express tagged or mutant isoforms at endogenous levels. In addition, they pave the way for difficult experiments such as tissue-specific allele switching and functional analysis in post-mitotic or polyplloid cells. Therefore, our method retains the advantages of homologous recombination while capitalising on the mutagenic power of CRISPR.

KEY WORDS: Drosophila, Functional genomics, Gene targeting, Homologous recombination

INTRODUCTION
In mice, gene targeting is achieved by homologous recombination in embryonic stem (ES) cells, which are amenable to straightforward selection (Capecchi, 2005). In most other model species, no ES cells are available and, therefore, alternative approaches are needed. Golic and collaborators were the first to describe protocols for gene targeting by homologous recombination in Drosophila (Gong and Golic, 2003; Rong and Golic, 2000). Soon thereafter, gene targeting by ‘ends out’ homologous recombination was used to delete genomic fragments while at the same time introducing exogenous sequences with base-pair precision (Gong and Golic, 2003; Huang et al., 2009; Huang et al., 2008; Liu et al., 2012; Xie and Golic, 2004). Current protocols for homologous recombination involve several key steps (Gong and Golic, 2003; Huang et al., 2009; Huang et al., 2008; Weng et al., 2009; Xie and Golic, 2004; Zhou et al., 2012). First, homology arms, typically 3-5 kb, are ligated into the targeting vector, which is then inserted at a random location in the genome by P-element mediated transformation. As the targeting vector comprises FLP recombination targets (FRT) and I-SceI restriction sites, it can be released from the random genomic location and linearised to gain access to the target locus (Huang et al., 2009; Rong and Golic, 2000). The most commonly used targeting vector, pGX (Huang et al., 2009), comprises two key features between the homology arms (the region that becomes inserted at the locus): mini-white, which serves as a genetic marker; and an attP site to enable PhiC31-mediated insertion of exogenous sequences in the targeted locus (Bateman et al., 2006; Bischof et al., 2007; Groth et al., 2004; Venken et al., 2006). The mini-white genetic marker is flanked by LoxP sites so that it can be removed, leaving only the attP site (and one LoxP site) at the modified locus. Outside the homology arm, on the 3’ end, pGX contains a DNA fragment that expresses Reaper, a pro-apoptotic protein, when Gal4 is present. This part of the vector should not integrate in the targeted locus following accurate homologous recombination and therefore allows the negative selection of illegitimate events by crossing to a suitable Gal4 driver. So far the promises of this feature have been only partially realised because of the unavailability of Gal4 drivers that fulfil the dual conditions of being a strong activator in essential tissues while not being marked with mini-white so as not to interfere with the mini-white of the targeting vector. Moreover, the frequency of successful targeting events has remained frustratingly low. Typically, 20,000 to 100,000 flies must be screened to identify one successful targeting event (Huang et al., 2009; Huang et al., 2008; Zhou et al., 2012). Despite the palpable benefits of homologous recombination, particularly the opportunity to insert an attP site at any genomic location, the low frequency of recombination and the large number of false positives have deterred many laboratories from initiating homologous recombination projects.

Recently, the advent of sequence-specific endonucleases such as transcription activator-like effector nucleases (TALENs) and the RNA-guided DNA endonuclease Cas9 has dramatically opened opportunities for creating double-strand breaks (DSBs) in the genomes of yeast, flies, zebrafish and cultured mammalian cells (Bassett et al., 2013; Bedell et al., 2012; Chang et al., 2013; Cho et al., 2013; Cong et al., 2013; DiCarlo et al., 2013; Gaj et al., 2013; Gratz et al., 2013; Hwang et al., 2013; Liu et al., 2012; Mali et al., 2013). As DSB-induced non-homologous end joining (NHEJ) creates deletions, TALENs and CRISPR are effective site-specific mutagenic tools. However, these deletions are variable in length and not amenable to subsequent genome engineering. This can be overcome with concurrent introduction of homologous sequences so that the DSB is repaired by homologous recombination while exogenous sequences are inserted at the targeted locus. Proof-of-principle that this can be achieved in Drosophila has recently been reported (Gratz et al., 2013). DSBs and homologous recombination...
were induced by co-injecting CRISPR components (guide RNA, and a Cas9-encoding plasmid), along with template DNA comprising short (50 bp) homology arms flanking an attP site, into Drosophila embryos. Successful integration of the attP site was demonstrated although, because of the absence of a genetic marker, successful recombinants had to be selected by PCR from pools of animals derived from injected embryos, adding a substantial amount of work to the protocol. It must be also kept in mind that CRISPR is somewhat restricted in the sequences it can target (Mali et al., 2013) and can cause off-target effects (Fu et al., 2013), a problem that could be confounded when small homology arms are used (50 bp). The results of Gratz et al. (Gratz et al., 2013) nevertheless suggest that combining CRISPR with homologous recombination is a promising approach to genome engineering. As an alternative, DSBs and homologous recombination could be induced with TALENs (Beumer et al., 2008). However, the production of plasmids encoding TALENs still requires substantial bench work, despite recent progress (Cermak et al., 2011), tempering enthusiasm for this option, at least in its current state.

We have devised vectors and protocols for homologous recombination in Drosophila, which are effective with and without concurrent CRISPR-induced DSBs. One CRISPR-independent approach, which requires prior integration of the targeting vector by P-element mediated, achieved a ~10-fold frequency improvement over the most recent CRISPR-independent protocol (Huang et al., 2009; Zhou et al., 2012) and reduced the number of false positives to very few. In the second approach, also CRISPR independent, the targeting vector was injected in embryos and homologous recombinants were identified in the F2 progeny with an eye colour marker screen. In the third approach, embryos were similarly injected with the targeting vector but this time with CRISPR components. Approach 1 is reliable and does not suffer with the uncertainties associated with CRISPR; it is therefore currently the safest while still achieving excellent frequencies. With the third protocol come the advantages – and current uncertainties – of CRISPR. Importantly, all three protocols use the same targeting vector, which comprises a Cre-removable reporter gene and selectable marker, as well as an attP site. To take full advantage of the latter, we have created a set of reintegration vectors that enable a variety of genetic manipulations including tissue-specific allele switching and the generation of genetic mosaics in post-mitotic or polyploid tissues.

RESULTS
High-frequency integration of a multifunctional DNA fragment at specific genomic locations
As a benchmark, we first tested the most commonly used Drosophila targeting vector (pGX) and protocol (Huang et al., 2009) with rhogap102A (a gene located on the 4th chromosome of Drosophila). The lengths of the homology arms were 5 kb (5‘ arm) and 3 kb (3‘ arm). From 80,000 screened flies, 365 white+ flies were identified. They were crossed a second time to 221-Gal4[w-], with the aim of eliminating false positives missed in the first round. This left us with 170 candidates to test by PCR with primers spanning the homology arms. Of these, only one turned out to represent a true homologous recombinant. The high number of false positives is in accordance with what was previously described for other genes (Huang et al., 2008; Huang et al., 2009). To overcome the problem of false positives, we generated ubiquitin-Gal4[3xP3-GFP], which was predicted to activate UAS-reaper throughout the larva and not only in the nervous system as 221-Gal4[w-]. This driver was inserted in a vector that uses a fluorescent genetic marker (3xP3-GFP, expressed in the eye and ocelli (Horn et al., 2000). As the 3xP3-GFP maker does not interfere with that of the targeting vector (mini-white), both constructs can be tracked in the same animal. None of the 364 false positives obtained during the above targeting of rhogap102A survived in the presence of this driver, showing its effectiveness at eliminating false positives. We next set out to improve the targeting vector, retaining important features of pGX such as mini-white and the attP site, and adding a reporter gene (Cherry) to enable rapid assessment of gene expression following gene targeting (Fig. 1A). This vector, which we call pTVCherry, was used to re-target rhogap102A, using the same homology arms as before. Donor flies (carrying the targeting vector at a random location) were crossed to flies expressing FLP and I-SceI (Fig. 1B) and about 200 progeny with mottled red eyes (i.e. carrying both the targeting vector and hs-FLP, hs-I-SceI) were collected. These were crossed to ubiquitin-Gal4[3xP3-GFP] to eliminate unmobilised donor chromosomes as well as false positives (Fig. 1B). Red-eyed flies resulting from this second cross were considered candidate knockouts (KOs). In this instance, two such candidates were identified from about 8000 flies examined and one was confirmed to be a homologous recombinant by PCR (Table 1). Therefore, a two-generation protocol (Fig. 1B) followed by cursory examination of about 8000 flies were sufficient to generate one KO. To confirm the effectiveness of this protocol, five more genes were targeted with the same strategy (Table 1; supplementary material Figs S1 and S2). In all cases, 200 females carrying the targeting vector and hs-FLP, hs-I-SceI were used and, on average, a confirmed KO was identified after screening 3000 flies (range 1000-8000). This is equivalent to a 10- to 100-fold frequency increase over previously reported results (Huang et al., 2009; Zhou et al., 2012). The number of false positives was low, approximately equal to the number of true homologous recombination events (Table 1). The small number of false positives can be directly attributed to the broad expression of ubiquitin-Gal4[3xP3-GFP]. The high targeting efficiency is likely due to features of pTVCherry as the same homology arms were used to target rhogap102A with pGX (frequency=1/80,000) and pTVCherry (frequency=1/8000). The reason for this difference is unknown but pTVCherry still appears to be 10 times more conducive to homologous recombination than pGX. This, along with an effective means of eliminating false positives, significantly facilitates homologous recombination in Drosophila.

Accelerated gene targeting by direct injection, with or without CRISPR
So far, homologous recombination in Drosophila has necessitated prior insertion of the targeting construct at a random genomic location before releasing it through genetic crosses (as described above). Targeting would be significantly accelerated if homologous recombination occurred in the germine of injected embryos. The high targeting efficiency of pTVCherry obtained with the crossing scheme suggested that it might be possible to achieve homologous recombination following injection of the targeting vector in embryos (Fig. 1C). Five different targeting plasmids were injected in the posterior region of pre-cellular embryos, along with a vector expressing I-SceI under the control of the germ line-specific vasa promoter to trigger linearization. For historical reasons, this vector (vasa-FUS) also expresses FLP and Utrophin-GFP, although these proteins are not expected to be relevant in the present protocol. In these experiments, 3000 white1118 mutant embryos were injected by Rainbow Transgenics, and the resulting adults were backcrossed to white1118 flies. The subsequent F2 progeny were screened for red eyes as this would indicate germ line transmission of the targeting
For three out of the five constructs, accurate targeting was confirmed by PCR (Table 1; supplementary material Fig. S1). Therefore, pTVCherry is compatible with two strategies, one of which can be completed in 6 weeks (Fig. 1B,C).

As DSBs stimulate homologous recombination, we asked whether the frequency of targeting by injection could be increased by co-injecting a sequence-specific endonuclease. We chose CRISPR/Cas9 to induce DSBs because of the ease of construction of the necessary plasmids. Embryos were injected with a plasmid encoding Cas9 and another plasmid encoding a wingless-specific target gRNA, along with the pTVCherry[wingless] and the plasmid encoding FLP and I-SceI (Fig. 1C). In three independent experiments, confirmed homologous recombinants, which were easily recognised by the presence of mini-white and the pattern of Cherry expression, were obtained at a frequency of 1 per 500 injected embryos for wingless (Table 1). Although more genes will need to be targeted before an average frequency can be obtained, this result, along with the finding that CRISPR efficiently induces DSBs in Drosophila (Gratz et al., 2013), suggests that, in many cases, CRISPR will be an effective tool to increase the frequency of homologous recombination. Finally, we tested whether the length of the homology arms could be reduced. One homologous recombinant in wingless was retrieved following injection of a targeting vector containing 500 bp homologous arms and the CRISPR components. This result suggests that the long homology arms we used in previous experiments may not be necessary.

**Post-targeting features and reintegration vectors**

Besides the high frequency of homologous recombination, a key feature of pTVCherry is the transcriptional reporter activity provided by Cherry and hence the potential to quickly confirm true targeting events, as illustrated in Fig. 1E for hedgehog. Another important
benefit is the attP site. To expand the range of genetic manipulations that the attP site affords, we designed a range of reintegration vectors (Figs 2, 3; supplementary material Table S1). Using pGE-attB (Huang et al., 2009) as a starting point, we created RIVwhite to allow reintegration of any cDNA (e.g. a wild-type form to confirm that the mutant phenotype associated with the KO is indeed due to destruction of the targeted gene). As RIVwhite uses mini-white as a genetic marker, it requires Cre-mediated removal of mini-white and associated sequences from the targeted locus before reinsertion (supplementary material Fig. S3, Table S1). To avoid this requirement, we also constructed RIVCherry, which has the same polylinker but uses 3xP3-Cherry as selectable marker (Fig. 2; supplementary material Fig. S3, Table S1). Thus, rescue by reintegration can be performed as soon as homologous recombination has been achieved (supplementary material Fig. S3). Using this procedure, reintegration of a wingless cDNA (including the 5' and 3' UTRs) in the wingless KO led to full rescue (not shown), suggesting that the mini-white and associated sequences remaining in the locus did not interfere with gene regulation. In the event that the cDNA did not rescue in this way, these exogenous sequences could easily be removed, along with the selectable marker of RIV, after reintegration (supplementary material Fig. S3). With

### Table 1. Frequency of gene targeting by crossing scheme and direct injection

<table>
<thead>
<tr>
<th>Gene</th>
<th>Confirmed KOs/ total flies screened</th>
<th>False positives</th>
<th>Confirmed KOs/ injected embryos</th>
<th>False positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhoGap102A</td>
<td>1/8000</td>
<td>1</td>
<td>0/3000</td>
<td>1</td>
</tr>
<tr>
<td>wingless</td>
<td>2/6000</td>
<td>0</td>
<td>2/3000</td>
<td>2</td>
</tr>
<tr>
<td>w/CRISPR</td>
<td>NR</td>
<td>NR</td>
<td>3/1500</td>
<td>0</td>
</tr>
<tr>
<td>w/CRISPR+500</td>
<td>NR</td>
<td>NR</td>
<td>1/500</td>
<td>0</td>
</tr>
<tr>
<td>hedgehog</td>
<td>2/4000</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>β-Catenin (arm)</td>
<td>2/4500</td>
<td>2</td>
<td>0/3000</td>
<td>3</td>
</tr>
<tr>
<td>dredd</td>
<td>2/6000</td>
<td>0</td>
<td>3/3000</td>
<td>2</td>
</tr>
<tr>
<td>sickle</td>
<td>2/2000</td>
<td>0</td>
<td>1/3000</td>
<td>1</td>
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Targeting by the crossing scheme was conducted as described in the legend of Fig. 1. Targeting by direct injection was achieved by injecting 3000 embryos (performed by Rainbow Transgenics). CRISPR-aided targeting was attempted for wingless, using the same homology arms and targeting vector as with the crossing scheme. This was carried out in three separate experiments involving 500 embryos each (one in house and two by Rainbow Transgenics). Each experiment led to one confirmed homologous recombinant. Finally, a targeting construct with 500 bp wingless-specific homology arms was injected along with CRISPR components (w/CRISPR+500) and one homologous recombinant was recovered following injection of 500 embryos. NA, not attempted; NR, not relevant.

Fig. 2. A set of reintegration vectors (RIV): diagrams and features. (A) The key features of the reintegration vectors described in this paper. The restriction sites available for cloning (MCS) are listed below. Some of the vectors use mini-white and others use 3xP3-Cherry (pax-Cherry) as genetic markers. (B, C) Low-magnification fluorescence micrographs show the suitability of 3xP3-Cherry as a larval (B) and adult (C) markers. RIV\textsuperscript{FRT}\textsuperscript{Wg.FRT}NRT\textsuperscript{HA-Wg/Cyo[white+]} animals are shown.
RIVCherry or RIVwhite, one can also re-integrate a tagged form of the cDNA, thus providing an assay for protein localisation without the need for an antibody, as illustrated for HA-tagged Wingless (Fig. 3A-C, supplementary material Table S1). If reintegration of the tagged cDNA rescues the mutant phenotype, as is the case for wingless, one can be confident that the tag does not significantly affect protein function and that its distribution is likely to reflect endogenous protein localisation. No transcription termination signals affected exon is shown as a hatched box, untranslated sequences are in grey and the region deleted by homologous recombination is marked by a red box.

DISCUSSION

We have described a vector that achieves high efficiency homologous recombination and provides immediate reporter gene expression in larval muscles (Fig. 3D). As an additional tool to control gene expression in defined patterns, we also created another tag in the second position) could also be potentially used to estimate protein turnover or trafficking between different subcellular compartments. Importantly, this can be achieved at physiological levels of expression under the control of the endogenous promoter.
activity while retaining previously described features such as an easy-to-select genetic marker and an attP site. We have also improved on previous technology by devising a means of eliminating most illegitimate recombination events. Our targeting vector is compatible with three experimental strategies. One involves a cheap and effective two-generation crossing scheme following prior P-element-mediated transformation. The second involves embryonic injection of the targeting vector, along with an I-SceI-encoding plasmid, and selection in the F2 progeny. One might argue that I-SceI could be omitted from this protocol if the targeting vector were linearized prior to injection. However, in preliminary experiments, homologous recombinants were not obtained with linearized targeting vector (not shown). Moreover, previous experiments with small linear templates produced conflicting results (Beumer et al., 2008; Gratz et al., 2013). Because of these uncertainties, we have continued to inject supercoiled targeting vectors along with the I-SceI-encoding plasmid. Our third approach relies on the recent demonstration that DSBs stimulate homologous recombination in Drosophila (Beumer et al., 2008; Gratz et al., 2013). For this approach, we opted to induce DSBs with CRISPR and therefore co-injected four plasmids: the targeting vector, along with plasmids encoding a gene-specific guide RNA, Cas9 and I-SceI. We have chosen to express the guide RNA from a plasmid because this is more stable than RNA, an important consideration if the injection mix needs to be shipped to injection service providers. Importantly, our targeting vector is compatible with all the approaches described here so that if unexpected problems arose with CRISPR (e.g. off-target effects or recalitritant genes) or if the generation of TALENs became much easier, our protocol could easily be adjusted without the need for new targeting constructs.

Despite the versatility of our targeting vector and protocols, there is undoubtedly room for further improvement. A seemingly higher frequency of CRISPR-aided homologous recombination was reported recently (Gratz et al., 2013). However, in this report, only a small double-stranded oligonucleotide was integrated, and recombinants therefore had to be selected by PCR from pools of candidate animals, a relatively laborious procedure. We suggest that the ease of screening with a visible eye marker, combined with the reporter activity of pTVCherry more than offsets the need to increase the number of embryos to inject. Nevertheless, it is likely that frequency improvements are possible. One possibility would be to create two DSBs instead of one (as reported by Gratz et al., 2013). This could be easily achieved by including a second gRNA-encoding plasmid in the injection mix. In addition, it is conceivable that the frequency of CRISPR-aided homologous recombination could be increased by using vasa-Cas9 transgenic embryos as injection hosts or by adjusting the relative concentrations of the various injected plasmids. We have generated transgenic vasa-Cas9 flies but they have yet to be tested. Targeting frequency might also be increased by introducing the guide RNA directly as RNA and not via a plasmid. However, as mentioned in results, we favour DNA injection because it can readily be outsourced. Another area of possible improvement concerns the size of homology arms as the relatively long arms (3-5 kb) that we have used in this study can be difficult to handle. As one experiment suggests (see Results), it is likely that 500 bp arms might suffice, although this will need further validation. We do not favour reducing the size further as this could lead to loss of specificity. Despite all the above suggestions for improvement, as they stand, our vector and protocols should help transform gene targeting by homologous recombination into a routine procedure in Drosophila, and perhaps in insect species that lack extensive genetic resources such as Anopheles or Tribolium.

Much of the genome editing possibilities described above are focused on the coding region. We have targeted the ATG-containing exon to preserve downstream regulatory elements (often found in the first intron) thus ensuring correct expression of reintegrated cDNAs. In all the cases we have studied so far, insertion of Cherry and mini-white did create a null allele. However, one must be aware that, following Cre-mediated excision of these elements, a truncated protein could become expressed from a downstream coding exon and restore partial function. Any such concern could be allayed by increasing the size of the deleted fragment appropriately. Another potential drawback of our approach is that the ‘rescued locus’ is not identical to that of the wild type. However, as long as full phenotypic rescue is achieved after reintegration, one can be confident that the subsequent analysis will be physiologically relevant. Although our targeting vector has been used so far to delete expressed exons, it is theoretically suitable for engineering any genomic sequence, including regulatory regions and enhancer elements. We envision that our targeting approach could be used routinely to study 1-2 kb regulatory element (a size that we have reliably deleted by homologous recombination). Large genomic regions (e.g. 20 kb) could be also engineered by two successive targeting operations to flank the region of interest with one attP site at each end. Mutated variants could then be reintegrated by recombination-mediated cassette exchange (RMCE) (Oberstein et al., 2005; Venken et al., 2011).

We have designed reintegration vectors that enable a variety of post-targeting applications. The basic reintegration vectors allow the insertion of a diverse set of reporters by co-injection with a plasmid encoding the PhiC31 integrase (Bateman et al., 2006; Bischof et al., 2007; Groth et al., 2004; Venken et al., 2006). They also provide a means of rapidly assessing the subcellular localisation and abundance of the protein product, as the reintegrated tagged cDNA should be expressed under endogenous control. Moreover, any targeted locus can be made to express Gal4, Gal80 and QF, thus widening the scope for sensitive reporter assays or misexpression studies. Finally, the combination of our FRT-containing reintegration vectors and the high efficiency of FLP-mediated cassette excision facilitate the removal of gene function in post-mitotic or polyploid cells at any time and in a tissue-specific manner. This, along with allele switching, opens up assays that have so far been nearly impossible.

MATERIALS AND METHODS

General Drosophila genetics

All the standard fly strains are described at http://flybase.org and all experiments were conducted at 25°C unless otherwise indicated. To induce clones in the domain of hedgehog expression, y w hsp70-FLP UAS-GFP QUAST-tomato; RIVFRT.HhOllas.FRT QF larvae were cultured at 37°C for 50 minutes, 60±12 hours after egg laying. The resulting larvae were fixed and imaged 48 hours after the heat shock.

Immunostaining and microscopy

The following primary antibodies were used: rabbit anti-HA (1:1000, Cell Signaling Technology, C294F9) and mouse anti-Wingless (1:100, Hybridoma Bank). Secondary antibodies labelled with Alexa 488 or Alexa 555 (used at 1:200) were obtained from Molecular Probes. Imaginal discs were mounted in Vectashield with DAPI (Vector Laboratories). Fluorescence micrographs were acquired with a Leica SP5 confocal microscope. Embryo cuticles were prepared according to a standard protocol (Alexandre, 2008). Bright-field images from embryo cuticles were obtained with a Zeiss Axioaphot2 microscope with an Axiocam HRC camera. Bright-field and confocal images were processed with Photoshop CS4 (Adobe).
Plasmid design and features

All PCRs were performed with Q5 High-Fidelity polymerase from New England Biolabs (NEB, M0492L). DNA synthesis was performed using Genewiz or Integrated DNA Technologies (IDT). The plasmids constructed for this study (most of which will be deposited at the DRCG, Indiana) were: the targeting vector, pTVCherry; a plasmid expressing ubiquitous Gal4 (150 ng/μl), U6-target-gRNA (150 ng/μl); all as supercoiled plasmids. We have obtained evidence that, using this protocol, 500 bp homology arms might suffice.

Gene targeting

For targeting with the crossing scheme, the targeting vector, which was modified to contain the appropriate homology arms (here 3-5 kb arms were used but smaller arms are likely to suffice), was introduced at a random genomic locations by P-element-mediated transformation (Rainbow Transgenics, Bestgene or in house). Transformants (not necessarily mapped or homozygosed) were crossed to Bloomington stock flies and red-eyed groups to obtain the required flies (Bloomington stock flies). In most, but not all, cases screening was stopped after a homologous region had been identified. In all cases, we used the presence of GFP as a marker to identify the transgene carrying the corresponding allele. Details on their construction are provided in supplementary material Appendix S1.

<table>
<thead>
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<th>Plasmids</th>
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<tr>
<td>DGRC, Indiana: pTVCherry, vasa-FUS, U6-sickle-gRNA; a plasmid for expression of any gene-specific gRNA</td>
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<tr>
<td>England Biolabs (NEB, M0492L). DNA synthesis was performed using</td>
<td></td>
</tr>
<tr>
<td>All PCRs were performed with Q5 High-Fidelity polymerase from New</td>
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<tr>
<td>Subsequent screening was performed after PCR (550 bp) using a suitable BAC or genomic DNA as a template and ligated into multiple cloning sites of pTVCherry to generate pTVCherry[GeneX].</td>
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</tr>
<tr>
<td>Prepare injection mix comprising (all plasmids), pTVCherry[GeneX] (600 ng/μl), U6-geneX-gRNA (150 ng/μl), vasa-FUS (200 ng/μl) and vasa-Cas9 (150 ng/μl), and inject (can be subcontracted) into white mutant embryos. If funds permit, generate in parallel pTVCherry[GeneX] transfectants by P-element-mediated transformation. These flies could be used in a backup CRISPR-independent protocol, as described in Fig. 1B.</td>
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<tr>
<td>Cross all the adults arising from injected embryos individually or in small groups to white mutants and screen for white’ flies in the progeny (can also be subcontracted).</td>
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</table>

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Sebastian Gerety (Sanger Institute) provided CRISPR/Cas9-related advice and tools. Konrad Basler (Zurich), Chris Potter (Johns Hopkins), Yang Hong (Pittsburgh) and Sean Megason (Harvard) provided plasmids.

Competing interests

The authors declare no competing financial interests.

Author contributions

L.A.B.-L. and C.A. A.M. and L.P. contributed important experimental results. The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl; doi:10.1242/dev.100933/-/DC1

References


Appendix S1

Details on plasmid construction.

*Targeting vector pTVCherry: Two modules were sequentially introduced in pGX-attP (Huang et al., 2009). The first module contained mFRT71-Gal4-hsp70polyA created by PCR with the primers mFRT71Gal4F (5’ gatcgcaccggtgaagtttctatactttctagagaacACTgaagctactgtacttcagacacaagc 3’) and AgepAR (5’ gatcgcaccggtgatctaaacgagtttttaagcaaactccctcc3’), using pGAwB (Brand) as a template. This was cloned in the unique AgeI site of pGX-attP. The second module contained a variant FLP recombination target (mFRT71) (Hadjieconomou et al., 2011) followed by myrCherry-SV40polyA. This was created by PCR with the primers FRT71CHEF (5’ gatccggcggccgcagttttcatctcagagctacttcagacacaagc 3’) and CHEpAR (5’ gatcgcactgctacgcttgggctgcaggtcgacctcgagg 3’), using pCS-memb-mCherry (gift from Sean Megason, Harvard University) as a template. The PCR product was inserted in the plasmid obtained from the previous step as a BsiWI fragment. The resulting plasmid (pTVCherry) was verified by sequencing and found to contain no mutation. Nevertheless, no Gal4 activity could be detected (for unknown reason since no mutation was detected). This is a minor inconvenience because Gal4 can be reintegrated in the targeted locus by injection of RIVGal4 (see below). For each targeting experiment, pTVCherry was modified by introducing gene-specific homology arms in the multiple cloning sites (see diagram in Fig. 1A).

*ubiquitin-Gal4[3xP3-GFP]: UAS-reaper was included in the targeting vector in such a way that it becomes lost upon homologous recombination (and retained following most illegitimate events). Thus, many false positives should be eliminated by crossing to a suitable Gal4 driver. One restriction is that the driver be not marked with mini-white since this would prevent the targeting vector from being tracked. The Gal4 driver used so far, 221-Gal4[w-] (Bloomington stock 26259), is weakly expressed and therefore ineffective at eliminating many false positives. We have therefore constructed a new driver that is robustly expressed in all tissues and is not
marked by mini-white. The hsp70 promoter of pCaSpeR-hs was replaced by the ubiquitin promoter (Xho1-Xba1) to generate CaSpeR-ubi. Next the mini-white gene was mutated by digest with Nco1 and EcoRV followed by religation. 3xP3-GFP (cassette provided by K. Basler, University of Zürich) was then inserted between SacI and PstI to generate CaSpeR-ubi-3xP3-GFP. Finally, a Gal4 cDNA was introduced as a NotI-BglII fragment to generate ubiquitin-Gal4[3xP3-GFP].

*RIVwhite*: This was made with pGE-attB as a starting point (Huang et al., 2009). The SphI site was removed since it contains a translation initiation codon that could potentially interfere with the expression of reintegrated genes. This was achieved by EcoRI-KpnI digestion followed by insertion of a double stranded oligonucleotide containing the following sequence between EcoRI and KpnI:
tccggacggccgagtctctactatatttaataacactagtctcagtggcgcgcc.

*RIVGal4*: This was generated by inserting a Gal4 cDNA with hsp70’s polyA as a NotI fragment (isolated from pGAwB) into the NotI site of RIVwhite.

*RIVFRT.MCS2.pA.FRT QF*: All the relevant features of this vector were synthesized. Note the presence of hsp70’s polyA immediately downstream of MCS2. The QF message is terminated with the SV40 polyA.

*RIVCherry*: This was generated by inserting a double-stranded oligonucleotide containing a multiple cloning site (MCS, sites listed in Fig. 3) in RIVFRT.MCS2.FRT QF with all the sequences between the attB and the LoxP sites excised with BsiWI and AgeI. This construct contains no polyA site immediately downstream of the MCS.

*RIVQF*: This was generated by removing the FRT cassette from RIVFRT.MCS2.FRT QF with BsiWI and AvrII, followed by blunt end religation.

*RIVGal80*: RIVCherry was cut with NotI and Xho1 to insert a NotI-Xho1 Gal80-SV40polyA fragment obtained from Tubulin-Gal80 (gift from Liqun Luo, Stanford University)
*RIV\textsuperscript{FRT,MCS,pA,FRT MCS}\textsuperscript{3}*: QF was excised with AvrII and AgeI from RIV\textsuperscript{FRT,MCS2,pA,FRT}QF and MCS3 was inserted as a double stranded oligonucleotide. Note the presence of the \textit{hsp}70polyA immediately downstream of MCS.

*RIV\textsuperscript{FRT,MCS1,FRT}*: MCS1 was inserted as a double stranded oligonucleotide into RIV\textsuperscript{white} digested with EcoRI and Ascl. This construct contains no polyA site immediately downstream of the MCS.

*RIV\textsuperscript{FRT,HhOllas,FRT QF}*: The \textit{hedgehog} open reading frame was tagged in frame with an OLLAS epitope (Park et al., 2008) as follows: \ldots SH254-GG-SGFANELGPRLMGK \ldots \textit{hedgehog} 3’ UTR was inserted downstream and the combined \textit{hedgehog-Ollas-3’UTR} was inserted as a NotI-Ascl fragment into RIV\textsuperscript{FRT,MCS2,FRTQF}.

*RIV\textsuperscript{Wg-HA/white}*: A full length \textit{wingless} cDNA tagged with HA, along with 135 bp of 5’UTR and 1200 bp of 3’UTR was inserted as a NotI-Ascl fragment into RIV\textsuperscript{white}.

*RIV\textsuperscript{FRT,Wg,FRT NRT-HA-Wg}*: First, pMT-NRT-HA-Wg was made by replacing the NotI-BglIII fragment of pMT-Wg (Lawrence et al., 1995) with a synthetic fragment containing, from 5’ to 3’, the 5’UTR of \textit{wingless}, the \textit{neurotactin} open reading frame, DNA encoding two HA epitopes, and remaining \textit{wingless} coding sequences (up to the BglIII). The QF open reading frame was then excised from RIV\textsuperscript{FRT,Wg,FRT QF} with AvrII and AgeI and replaced by a Xbal-AgeI fragment containing NRT-HA-Wg (with 135 bp of 5’UTR and 1200 bp of 3’UTR ) obtained from pMT-NRT-HA-Wg.

*\textit{vasa-FLP.2A.Utrophin.2A.I-SceI (vasa-FUS)}*: This construct was made using, as template, the 3xP3-GFP/\textit{vasa-PhiC31} plasmid provided by K. Basler (University of Zuerich). The \textit{PhiC31} open reading frame was excised with NdeI and BssHII. A double stranded oligonucleotide containing NdeI, KpnI, Pmel, and BssHII was inserted to create 3xP3-GFP-\textit{vasa-PL-attB}. A single open reading frame encoding \textit{FLP-2A-Utrophin-GFP-2A-I-SceI} (Trichas et al., 2008) (each amplified separately with the primers below) was then introduced into 3xP3-GFP-\textit{vasa-PL-attB} to make \textit{vasa-FUS}.
NotFLP: 5’caccattgcggcggcatgccccacaatttgtatattag3’
BglIIFLPR: 5’aaccgtggctagcagctctctctgctctttatatgttggtaaggcaagg3’
GFPUtroF: 5’gaatcctaggcatggtgagcaagggcaggagt3’
GFPUtroR: 5’gccaatcgatgggtctatggtgacttgctgaggatgc3’
AvrIISeF: 5’ccacgaagagcctaggacccgggctagagcagctgcaacaaaaaagagagagggggtcgg3’
ApaISceR: 5’tacccagggccgctagctttcaggagagatgtttgtagg3’

* U6-BsaI-gRNA: DNA comprising the U6 promoter (Das et al., 1988), two BsaI restriction sites and guide RNA scaffold (Mali et al., 2013) was synthesized. The two BsaI restriction sites which flank an EcoRI site, are shown in red with the overhangs in blue. DNA encoding the gRNA scaffold is shown in ochre.

The relevant portion of the sequence is:

*U6-wingless-gRNA: This was made by inserting the double-stranded oligonucleotide recognizing the wingless target sequence into BsaI-digested U6-BsaI-gRNA.

wg forward : ttgaaggccgctgccagtgc
wg reverse : aaaccatggagccgtgacctt

*vasa-Cas9: A humanized Kpn1-Pmel cas9 cDNA (Mali et al., 2013) was inserted in the 3xP3-GFP-vasa-PL-attB described above.
REFERENCES


**Fig. S1. Molecular characterisation of 6 targeted loci.** Coding exons are represented in orange and non-translated exons are shown in grey. Red boxes indicate the deleted regions, along with the size of the deletion. The primers used to amplify the 5’ and 3’ homology arms are the top 4 primers shown in the table. The two primers listed at the bottom, which were the gene-specific primers used to confirm the KO, hybridise to the genome outside the homology arms. The ‘genomic, forward’ primer was used in combination with a reverse primer that hybridises with the attP site while the ‘genomic, reverse’ was used with a forward primer that hybridizes with the polyA signal downstream of the Gal4 cDNA. Agarose gels show the confirmation PCR fragments with red asterisks marking non-specific bands. The expected size of the 5’ homology arm is around 5 kb while that of the 3’ arm is around 3 kb.

**Fig. S2. Cuticle phenotypes of homozygous KO embryos for hedgehog (A), wingless (B) and β–catenin (C).** Low and high magnification images are shown.

**Fig. S3. Excision of genetic markers after gene targeting.** Structure of a generic targeted locus showing the sequences that are excisable with Cre. As shown on the left, excision can be performed immediately after targeting to remove the mini-white marker thus allowing insertion of a mini-white- or 3xP3-Cherry-marked reintegration vector. Alternatively, as shown on the right, a 3xP3-Cherry-marked vector can be reintegrated before Cre-mediated excision. Either route can lead to a locus that only carries the reintegrated cDNA with the attL (attP/B) product of integration at the 5’ and a LoxP site at the 3’.
<table>
<thead>
<tr>
<th>VECTOR NAME</th>
<th>VECTOR FEATURES</th>
<th>APPLICATIONS</th>
</tr>
</thead>
</table>
| RIV$^{\text{white}}$ | attB MCS mini-white | - Rescue with wt cDNA  
- Rescue with tagged cDNA  
- Reintegration of mutant forms  
- Reintegration of any cDNA |
| RIV$^{\text{Cherry}}$ | attB MCS Pax-Cherry | - All functions of RIV$^{\text{white}}$  
- Avoids mini-white marker  
- Reintegration without the need for Cre-mediated excision of cherry and white from targeted locus |
| RIV$^{\text{Gal4/white}}$ | attB Gal4 pA mini-white | - Gal4 activity in domain of targeted gene  
- Transcriptional reporter |
| RIV$^{\text{QF/Cherry}}$ | attB QF Pax-Cherry | All functions of RIV$^{\text{Gal4}}$ except that QF is the transactivator |
| RIV$^{\text{Gal80/Cherry}}$ | attB Gal80 pA Pax-Cherry | Repression of Gal4 in domain of targeted gene |
| RIV$^{\text{FRT.MCS1.FRT}}$ | attB $FRT$ MCS1 $FRT$ mini-white | - All functions of RIV$^{\text{Cherry}}$  
- Removal of rescuing cDNA in defined pattern  
- Mutant clones in post-mitotic and polyploid cells |
| RIV$^{\text{FRT.MCS2.pA.FRT QF}}$ | attB $FRT$ MCS2 pA $FRT$ transcriptional activator QF Pax-Cherry | - All functions of RIV$^{\text{Cherry}}$ and RIV$^{\text{FRT.MCS FRT}}$  
- Removal of rescuing cDNA in defined pattern with concomitant overexpression of any gene  
- Transcriptional analysis |
| RIV$^{\text{FRT.MCS.pA.FRT MCS3}}$ | attB $FRT$ MCS pA $FRT$ MCS3 Pax-Cherry | - All functions of RIV$^{\text{Cherry}}$ and RIV$^{\text{FRT.MCS FRT}}$  
- Allele conversion |

MCS: 5’ EcoRI, NotI, BgIII, Nhel, Pmel SpeI, XhoI, AscI, KpnI 3’  
MCS1: 5’ EcoRI, NotI, BgIII, Nhel, Pmel SpeI, XhoI, AscI 3’  
MCS2: 5’ EcoRI, NotI, BgIII, Nhel, Pmel SpeI, AscI 3’  
MCS3: 5’ AvrII, ClaI, Apal, PacI, NruI, AgeI 3’